May - June 8(3) : 338-46

Single-Chain Fv/Folate Conjugates Mediate Efficient Lysis of Folate-Receptor-Positive Tumor Cells

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Bispecific antibodies that bind to a tumor antigen and the T cell receptor (TCR) redirect cytotoxic T lymphocytes (CTL) to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the folate receptor (FR), is expressed on most ovarian carcinomas and some types of brain cancer. Recently, it was shown that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high-affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein. In this paper, it is shown that the size of these conjugates can be reduced to the smallest bispecific agent yet described (30 kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody KJ16. The scFv/folate conjugates are as effective as IgG/folate conjugates in mediating lysis of FR^+ tumor cells by CTL. The optimal folate density was in the range of 5–15 folate molecules per scFv or IgG molecule, which yielded half-maximal lysis values (EC_{50}) of approximately 40 pM (1.2 ng/mL for scFv). Finally, the scFv/folate conjugates could efficiently target tumor cells even in the presence of free folic acid at concentrations that are normally found in serum. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors and in reduced immunogenicity.

INTRODUCTION

It has been known for over 50 years that the immune system is capable of attacking and eliminating very large tumor burdens but sometimes fails to do so (*1*). Although the basis of this "escape" is incompletely understood (*2*), one mechanism involves the failure of tumor cells to express antigens in a context that is essential for recognition by the immune system [reviewed by Pardoll (*3*)]. Another mechanism might be the loss of costimulatory ligands and adhesion molecules that aid in the recognition and activation of T cells (*4*).

One potential way to direct T cells or other immune effector cells against tumor cells is with bispecific antibodies [reviewed by Fanger (*5*)]. Bispecific antibodies can be constructed to recognize two separate antigens, one on the tumor surface and the other on the surface of a cytotoxic T cell (e.g. TCR[1]). Many tumor cells have potential target antigens that are tumor-specific or quantitatively more abundant on tumor cells than normal cells (tumor-associated). By bringing together the tumor cell and an activated T cell, bispecific antibodies can redirect the cytotoxicity of T cells against tumors. Previous work has demonstrated the effectiveness of bispecific antibodies against tumors *in vitro* and *in vivo* and some clinical trials have been initiated (e.g. see refs 6–12). It has generally been agreed that optimizing the properties of bispecific antibodies should improve their clinical effectiveness.

Among the tumor antigens targeted with bispecific antibodies has been the high-affinity folate receptor (FR), also called the folate binding protein. The FR is now known to be expressed at elevated levels on many human tumors, including ovarian carcinomas [e.g. one study showed that 98% of ovarian tumors express the FR (*13*)], choroid plexus carcinomas, and ependymomas (*14*, *15*). These cancers affect a significant segment of the population: ovarian cancer is the fourth leading cause of cancer death among women (*16*) and at least 30% of early childhood tumors are diagnosed as ependymomal or choroid plexus tumors (*17*, *18*).

The presence of high levels of FR on human tumor cells has made it an attractive candidate for tumor-specific therapeutics. Monoclonal antibodies to the human FR have been generated and shown to be effective at targeting FR^+ tumors *in vitro* (*19–21*). Clinical trials with radiolabeled antibodies and anti-FR/anti-CD3 bispecific antibodies have recently been initiated (*9*, *10*, *12*). Another approach has been to use the endocytic properties of the FR to deliver toxins or antisense nucleotides to the interior of malignant cells (*22*, *23*). Although relatively low levels of FR mRNA have been detected in most normal human tissues (*14*), several studies have shown that normal choroid plexus, kidney, thyroid, colon, and placenta may have elevated levels [*14*, *24*, *25*; reviewed by Antony (*26*)]. Despite the presence of FR on normal human tissue, clinical trials using the anti-FR/anti-CD3 bispecific antibody have not demonstrated any toxicity associated with cyotolysis of normal FR expressing tissue (*9*, *10*).

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[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1997.

[1] Abbreviations: CTL, cytotoxic T lymphocyte; FR, folate receptor; TCR, T cell receptor; scFv, single-chain antibody binding domain; MHC, major histocompatibility complex; K_D, dissociation constant; SV40, simian virus 40; EC_{50}, concentration of antibody/folate conjugate required for half-maximal CTL mediated cytotoxicity; fol, folate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; FITC, 5-aminofluorescein isothiocyanate; Ga, gallium; Fab, antigen binding fragment derived from papain digestion of Ig molecule; V regions, variable regions of IgG heavy and light chains; ϵ, extinction coefficient; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.3; PBS-BSA, PBS containing 0.1% bovine serum albumin; MTX, methotrexate.

S1043-1802(97)00024-4 CCC: $14.00

The high intrinsic affinity of folate for FR ($K_d \sim 1$ nM) suggested to us that attachment of folate directly to an anti-TCR antibody might efficiently target FR^+ tumor cells for lysis by T cells. We recently reported that such conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein [$K_d \sim 1.5 \times 10^{-6}$ (*27*)], which is responsible for normal dietary uptake of folate and the transport of folate-based dihydrofolate reductase inhibitors such as methotrexate. It is reasonable to predict that the most effective agents for targeting solid tumors will have reduced sizes that allow greater tumor penetration. For instance, comparative biodistribution studies with ^{125}I-labeled IgG, $F(ab)_2$, Fab fragments, and scFv in human colon carcinoma xenografts in athymic mice demonstrated that scFv molecules penetrated tumor more rapidly, to a greater depth, and more uniformly than other forms of the antibody (*28*).

In this paper, a 29-kDa scFv of the anti-Vβ8 antibody KJ16 (*29*) was conjugated with folate and its targeting potential was evaluated *in vitro*. Cytotoxicity assays with these preparations showed that lysis of mouse FR^+ tumor cells was highly specific and correlated directly with FR density ($r = 0.93$). Comparison between folate-labeled-IgG and scFv demonstrated that both conjugates have nearly identical targeting efficiencies ($EC_{50} = 40$ pM), and lysis with scFv/folate could be detected at concentrations as low as 1 pM. Direct competition experiments with free folate demonstrated that the scFv/folate conjugate could effectively target FR^+ tumor cells even at folate concentrations above normal serum levels. The reduced size of the scFv/folate compared to other bispecific reagents as well as its high potency suggests that it has potential for *in vivo* therapy. In addition, the conjugate may serve as a model for the development of future novel bispecific agents that contain small ligands specific for tumor cell surface antigens.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies. The following DBA/2-derived tumor cell lines were maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal calf serum, 1.3 mM L-glutamine, 100 units of penicillin/mL, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol: Mel, murine erythroleukemia cell (*30*); La, a subline of Mel selected on low folate (*31*); L1210, a murine leukemia cell line (*32*); and F2-MTX^{r}A, a MTX-resistant subline of L1210 selected for increased expression of FR-β by growth on low folic acid (*33*). La expresses primarily the α isoform of folate receptor (FR), F2-MTX^{r}A expresses only the FR-β isoform, and L1210 expresses both α and β isoforms. CTL clone 2C, a $V\beta8^+$ alloreactive cell line specific for L^d (*34*), was maintained in the same RPMI medium supplemented with 10% (v/v) supernatant from concanavalin A-stimulated rat spleen cells, 5% methyl α-mannoside, and mitomycin C treated BALB/c mouse spleen cells as stimulators. KJ16 is a rat IgG antibody specific for the Vβ8.1−2 domains of the TCR (*35*) and was provided by Drs. Kappler and Marrack. KJ16 monoclonal antibody was prepared from tissue culture supernatant generated in a Vita-Fiber miniflow path bioreactor (Amicon) and concentrated by precipitating twice in 50% ammonium sulfate. KJ16 Fab fragments, FITC-labeled Fab fragments, and KJ16 scFv were generated and purified as described previously (*29*). Briefly, scFv was refolded from inclusion bodies, and monomeric scFv was purified by G-200 HPLC purification. Monoclonal antibody 30.5.7 is specific for the major histocompatibility complex (MHC) class I product L^d (*36*) and was prepared as ascites fluid and used without further purification in some cytotoxicity assays.

Preparation of Antibody/Folate Conjugates. Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously (*27*, *37*). Unless indicated, a 5-fold molar excess of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC, Pierce Chemical Co.) was added to folate (Sigma) dissolved in dimethyl sulfoxide. After 30 min at room temperature in the dark, a 20−700-fold molar excess of the EDC-activated folate was added to 0.1−0.5 mg of antibody in 0.1 M MOPS, pH 7.5. After 1 h at room temperature, the sample was either applied to a Sephadex G-25 column pre-equilibrated in 0.1 M MOPS or immediately dialyzed into phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.3). If passed over a G-25 column, the excluded-peak fractions were pooled and dialyzed against PBS. After dialysis, protein concentrations were determined using the bicinchoninic assay (Pierce) using a protein A purified intact mouse antibody as the standard. Antibody conjugates were also analyzed spectrophotometrically at 363 nm, and the density of the folate per antibody was calculated by dividing the molar concentration of folate on the conjugate ($A363/\epsilon_M$; $\epsilon_M = 6197\ M^{-1}$) by the antibody concentration. Folate densities obtained ranged from ~1 to ~120 folates/antibody. Conjugates were stored at 4 °C in the dark.

Mass Spectrometry. Mass spectra were obtained on a TofSpec using electrospray ionization. Samples were dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and concentrated to 10−25 pmol/mL. Analysis was performed by the Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois.

Folate Binding Assays. Binding assays were conducted by using ^{125}I-labeled folate (NEN; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq). Cells were washed with PBS containing 0.1% bovine serum albumin, pH 7.3 (PBS-BSA), to remove excess free folate present in the cell culture medium. Cells, labeled folate, and competitors were incubated in triplicate in 50 μL of PBS-BSA for 1 h at 37 °C. Incubation at 37 °C has previously been shown to produce levels of binding similar to that obtained with acid pretreatment (*27*). Samples were loaded into tubes containing 300 μL of oil [80% (v/v) dibutyl phthalate/20% (v/v) olive oil], and bound and free ligand were separated by a 3 s centrifugation at 12000*g*. Tubes were frozen and cut to allow the radioactivity in the cell pellet and supernatants to be quantitated separately.

T Cell Receptor Binding Assays. The relative affinity of the scFv/folate conjugates for the TCR was determined by a competition assay with 5-aminofluorescein isothiocyanate (FITC)-labeled KJ16 Fab fragments as previously described (*29*). In brief, various concentrations of antibody were added to triplicate sets of 6×10^5 2C cells in the presence of a constant amount of FITC-labeled Fab fragments. After a 30 min incubation on ice, the entire mixture (antibody + FITC-labeled Fab fragments + 2C cells) was passed through a flow cytometer without washing. Inhibition by various KJ16 preparations was measured by quantitating the decrease in bound fluorescence by flow cytometry (performed with a Coulter Electronics EPICS 752 at the University of Illinois Biotechnology Center). The concentrations of unlabeled antibody giving 50% inhibition (IC_{50}) were determined relative to the maximum fluorescence (in the absence of inhibitor) and the background fluorescence (in the presence of a large excess of intact antibody).

Cytotoxicity Assays. Tumor cells were labeled with

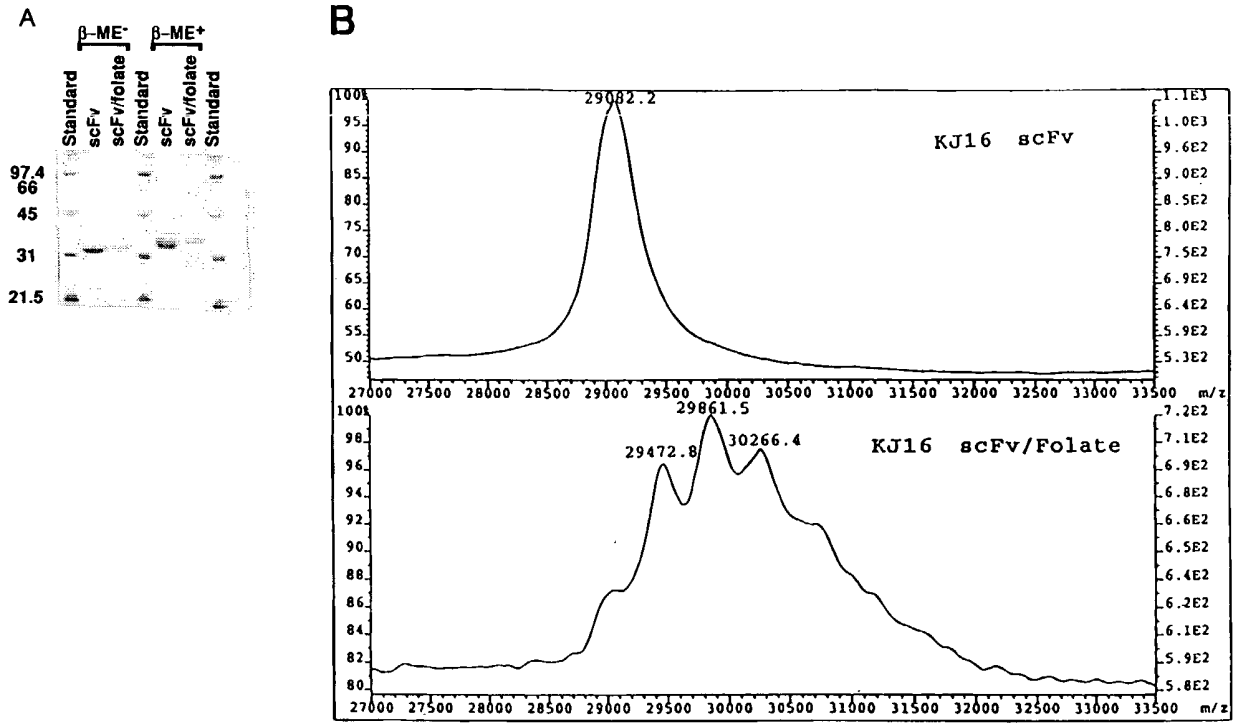

Figure 1. SDS–PAGE analysis and mass spectra of purified preparations of scFv KJ16 and scFv/folate conjugate: (A) samples were electrophoresed through a 10% polyacrylamide gel under reducing and nonreducing conditions, and proteins were visualized by staining with Coomassie Blue; (B) samples were concentrated, dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and mass spectra were obtained on a TofSpec using electrospray ionization. Purified scFv existed as a single species with a molecular mass of 29 082 Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular mass of a folate molecule (~400 Da), detectable up to 7 folates per antibody.

50–100 μL of ^{51}Cr (2.5 mCi/mL) for 60 min at 37 °C, washed twice with folate-free RPMI 1640 medium containing 5% (v/v) fetal calf serum (folate-free media), and used in 96-well plate cytotoxicity assays at 10^4 cells per well. Because each of these cell lines also expressed the alloantigen L^d, which is recognized by CTL 2C, assays were performed in the presence of anti-L^d antibody to minimize non-FR-mediated lysis. Ascites of anti-L^d antibody 30.5.7 was diluted 1:100 into folate-free media containing effector cells (2C). Effector cells were added to target cells at an effector-to-target cell ratio of 5:1. Antibodies and folate/antibody conjugates were diluted in folate-free media and added to triplicate wells at various concentrations. Plates were incubated at 37 °C for 4 h in 5% CO_2, and supernatants were removed for γ counting. For the inhibition of scFv/folate by free folate, a nonsaturating concentration of scFv/folate (3 nM) that would generate maximal cytotoxicity was used together with various concentrations of free folate. Unless indicated otherwise, the specific release mediated by the folate conjugates was determined by subtracting the release in the absence of the conjugates [e.g. % specific release = (experimental counts − spontaneous counts)/(maximal counts − spontaneous counts) × 100].

EC_{50} values (i.e. the concentration of antibody/folate conjugate required for half-maximal specific lysis) were determined by linear regression. Among independent experiments, EC_{50} values and maximum cytotoxicity could vary for the same conjugate preparation primarily due to differences in CTL activity. For example, noticeable reductions in CTL 2C activity can be observed after multiple passages *in vitro* (unpublished data, D.M.K.). Therefore, for comparison of EC_{50} values from different experiments (i.e. Figure 5B), assay results were normalized by dividing each calculated EC_{50} by the EC_{50} of the most potent conjugate in a given experiment [e.g. $EC_{50}^{\text{normalized}} = (EC_{50}/EC_{50}^{\text{low}})$]. The inverse of this normalized value, $[(EC_{50}^{\text{normalized}})^{-1}$, which we have called the targeting index] was plotted as a function of folate density on the antibody, where a targeting index = 1 specifies the most potent conjugate.

RESULTS

Characterization of scFv/Folate Conjugates. The scFv of KJ16, an anti-Vβ8 antibody, was purified from *Escherichia coli* inclusion bodies after guanidine denaturation, refolding, and HPLC gel filtration. Purified scFv migrated as an apparent 35-kDa protein on SDS–PAGE gels (Figure 1A). Folate was coupled to the scFv using the carbodiimide (EDC) reaction, which links carboxyl groups of folate to primary amine groups on the protein. In the engineering of the scFv, the V_L and V_H domains were joined by the 26 residue linker, 205s, that contains 8 lysine residues (*29*, *38*). We reasoned that the presence of multiple lysine residues in a highly accessible, solvent-exposed region may result in higher folate densities in the linker region as opposed to the antibody V regions. In initial studies, folate was coupled to the scFv at a 100:1 folate to antibody molar ratio. Under these conditions, scFv/folate preparations contained an average of 3–8 folates per antibody (N = 3), on the basis of spectrophotometric analysis. Consistent with this finding, migration of the scFv/folate conjugate on SDS–PAGE gels was slightly slower than that of scFv and the band was more diffuse (Figure 1A).

The folate density determined by photometry does not provide information about the heterogeneity of the conjugates. To determine the range of epitope densities within a single preparation, scFv/folate conjugates were

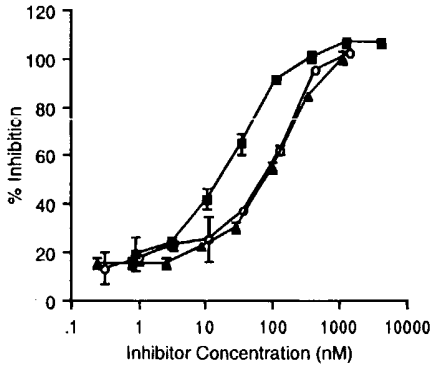

Figure 2. Binding of KJ16 scFv/folate to cell surface T cell receptor. The binding of FITC-labeled KJ16 Fab fragments to the Vβ8-positive T cell clone 2C was inhibited by purified scFv/folate, scFv, or Fab fragments. A total of 6×10^5 2C cells was incubated for 30 min at 4 °C with FITC-labeled KJ16 Fab fragments ($\sim 7.0 \times 10^{-8}$ M) and various concentrations of folate-labeled scFv KJ16 (7.8 fol/scFv, ▲), unlabeled scFv (■), and unlabeled Fab fragments (○). A relative affinity of the scFv/folate was determined by comparing the concentrations required to inhibit 50% of the FITC-labeled Fab fragments from binding the 2C TCR.

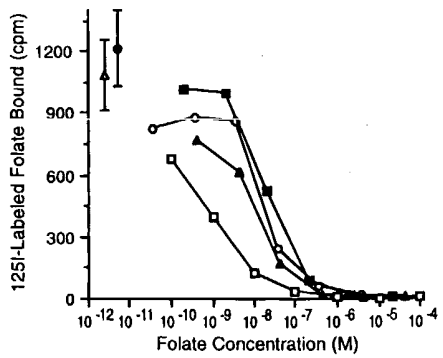

Figure 3. Binding of KJ16 scFv/folate to folate receptors. ^{125}I-labeled folate ($\sim 1.8 \times 10^{-10}$ M, 2000 Ci/mM) was incubated with F2-MTX^{r}A cells in the presence or absence of competitors for 1 h at 37 °C. Concentrations refer to folate rather than antibody concentrations. Competitors included free folate (□) and KJ16 scFv/folate conjugates with different folate densities: 2.8 fol/scFv (○), 9.2 fol/scFv (■), and 20.4 fol/scFv (▲). Inhibition was not observed in the absence of competitor (△) or in the presence of unconjugated scFv (●) (error shown is ± average SEM).

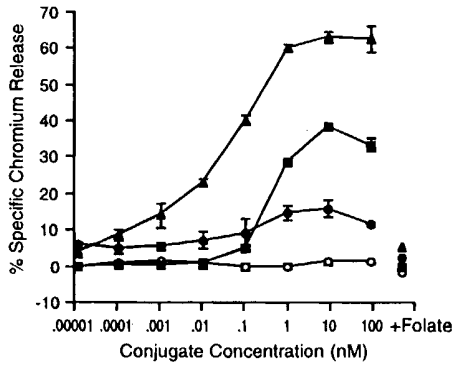

Figure 4. Cytotoxicity assay of various tumor cells lines with the scFv/folate conjugate and CTL clone 2C. Various concentrations of the scFv/folate conjugate were incubated with ^{51}Cr-labeled tumor cells and CTL 2C for 4 h at an effector-to target ratio of 5:1. Experiments were performed in the presence of anti-L^d antibody to minimize lysis due to recognition of L^d, the nominal ligand for CTL 2C. Lysis correlated directly with the level of FR expressed by the cell line: F2-MTX^{r}A (200 000 sites/cell, ▲); La (60 000 sites/cell, ■); L1210 (8 000 sites/cell, ●); Mel (not detectable FR, ○). Assays with free folate at a final concentration of 1.5 μM were performed with the scFv/folate conjugate at a concentration of 0.09 nM (+ folate).

examined by electrospray ionization mass spectrometry (Figure 1B). Unlabeled scFv demonstrated a single peak with a molecular mass of 29 082 Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular mass of a folate molecule (~400 Da), detectable up to 7 folates per antibody. Integration of mass spectra showed that >85% of the scFv molecules were labeled with one or more folate molecules. Folate densities estimated from mass spectra were generally 1.5–2-fold lower than densities estimated by spectrophotometry. This could in part be due to lower solubility of high-density conjugates under conditions required for mass spectrometry or to the dissociation of some folate molecules during ionization.

Binding of scFv/Folate Conjugates to the T Cell Receptor. To examine if folate conjugation affected the binding of scFv antibodies to the TCR, a scFv/folate conjugate was compared with unlabeled scFv. In a competitive flow cytometric assay, fluorescein-labeled KJ16 Fab fragments were inhibited from binding the Vβ8-positive CTL clone 2C by unlabeled Fab fragments, scFv, or scFv/folate (Figure 2). As shown previously, scFv antibodies have an approximate 3-fold higher apparent affinity than Fab fragments, possibly because of the presence of noncovalently associated scFv dimers (*29*). Comparison of folate-labeled and unlabeled scFv showed that the folate conjugate had an apparent affinity ~3-fold lower than scFv (i.e. approximately equal to KJ16 Fab fragments). This decreased binding, compared to unlabeled scFv, could be due either to chemical modification of active site residues with folate or to the interference of dimer formation by folate. The fact that folate conjugates bound the TCR as well as KJ16 Fab fragments, which have a K_D of ~130 nM (*29, 39*), indicated that the conjugates have potential to mediate lysis of target cells by CTLs.

Binding of scFv/Folate Conjugates to Folate Receptors on Tumor Cells. The ability of scFv/folate conjugates to bind folate receptors (FR) on the surface of tumor cells was examined by a competition binding assay using ^{125}I-labeled folate as the labeled ligand (Figure 3). The competition assay used the F2-MTX^{r}A cell line that expresses the β isoform of the FR. Competitors included various concentrations of free folate, unlabeled scFv, and three different scFv/folate preparations. Examination of the binding curves showed that folate-conjugated antibody, but not unlabeled scFv, binds to the FR$^+$ tumor cell line. However, on the basis of molar folate concentration, the folate conjugates had a relative affinity that was approximately 10–30-fold less than that of free folate. This decrease in apparent affinity was consistent with previous observations (*27*) and could be partly attributed to the carbodiimide labeling procedure. This procedure links folate through either the α or γ carboxyl group, but only linkage through the γ carboxyl retains binding (*40*). It is also possible that receptor-mediated internalization or decreased accessibility of the FR may also explain the lower apparent affinity of the folate conjugate. Included in the latter is the possibility that neighboring folates or amino acids sterically hinder interaction with the FR.

CTL-Mediated Lysis of FR$^+$ Tumor Cells by scFv/Folate Conjugates. The specificity and efficiency of tumor targeting with scFv/folate conjugates were examined in a ^{51}Cr release assay with CTL clone 2C (Figure 4). Four different tumor cell lines that have a range of cell surface FR densities were used as target cells: F2-

MTX^{r}A (200 000 sites/cell); La (60 000 sites/cell); L1210 (8 000 sites/cell); Mel (no detectable FR). Each of the FR$^+$ cell lines was lysed in the presence of the scFv/folate conjugate, and the extent of lysis was directly correlated with the expression level of the FR ($r = 0.93$). Lysis was completely inhibited by free folate, indicating that targeting of the tumor cells was specifically mediated by the folate receptor and not some other cell surface molecule. The lysis mediated by these conjugates was highly specific (e.g. the FR$^-$ cell line Mel was not lysed by the conjugate even at concentrations over 10^5 times that required for detectable killing of the FR$^+$ cell line F2-MTX^{r}A) and extremely potent (e.g. lysis was detectable at concentrations as low as 1 pM of scFv/folate). The slight reduction in observed lysis at the highest concentrations of conjugate occurs because excess bispecific antibody yields monospecific binding without cell-to-cell cross-linking (*41*). The presence of reduced folate carrier protein (as present in Mel and in all other normal cells) does not result in cell destruction. It is important to point out that the FR density reportedly on ovarian tumors is even higher (~1 million/cell) than those on these tumor cell lines (*21*).

Effects of Folate Density on Targeting. To examine the effects of folate density and labeling on the targeting efficiency of scFv/folate conjugates, the antibody was labeled with folate under various carbodiimide-mediated coupling conditions. The carbodiimide EDC couples folate through the free carboxyl groups, but when used in the presence of protein, it may also lead to protein modification and subsequent precipitation or inactivation. To evaluate the optimal levels of EDC for folate coupling, several different concentrations of EDC at a constant folate concentration were used during coupling. EDC used at either 13 or 65 mM generated conjugates with approximately equal targeting efficiency. EDC used at 260 mM yielded conjugates with reduced efficiency and frequently led to protein precipitation (data not shown).

To directly examine the effect of folate density, activated folate was prepared at a constant EDC/folate ratio (5:1) and conjugates were produced by adding different ratios of activated folate to the scFv protein. After dialysis to remove unreacted folate and excess EDC, conjugates were evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency. Folate densities ranged from approximately 1 to 20 folates per scFv. As shown in Figure 5A, each of the conjugates was capable of mediating lysis of the FR$^+$ tumor cell line by CTL clone 2C. However, conjugates with either the lowest density (1.3 folates/scFv) or highest density (20.4 folates/scFv) were 5–10-fold less effective than conjugates with intermediate folate densities.

To better evaluate the effects of folate density, the above experiment was performed with several additional folate conjugate preparations. To directly compare the results of these assays, the EC_{50} of each conjugate was determined by linear regression and divided by the EC_{50} of the conjugate that yielded the highest targeting efficiency (i.e. the lowest EC_{50}; see Experimental Procedures). A plot of the normalized targeting values as a function of the folate density (Figure 5B) demonstrates that optimal folate density appears to be in the range of 5–15 folates/scFv. At higher folate densities, the targeting efficiency of the conjugates is generally lower and more variable than those of conjugates with an intermediate amount of folate. The reduction in targeting efficiency at higher folate densities is not due to the inability of these conjugates to bind to FR$^+$ cells (Figure 3), but is likely a consequence of chemical modification

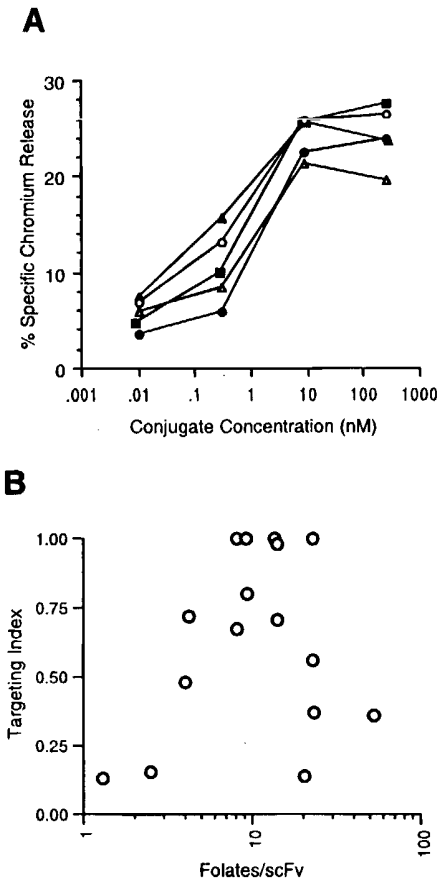

Figure 5. Effect of folate density on targeting with scFv/folate conjugates: (A) scFv/folate conjugates with different folate densities were prepared by labeling scFv protein with various amounts activated folate, using an EDC/folate ratio of 5:1 (EDC = 65 mM). Folate densities were 1.3 fol/scFv, ●; 4.0 fol/scFv, ■; 9.2 fol/scFv, ▲; 9.3 fol/scFv, ○; and 20.4 fol/scFv, △. Conjugates were tested in cytotoxicity assays using F2-MTX^{r}A and CTL 2C. (B) Relative targeting efficiency of various scFv/folate conjugates as a function of increasing folate density. Targeting index values were determined by normalizing EC_{50} values of the various conjugates with the most effective conjugate (value = 1) (see Experimental Procedures for details). Each open circle (○) represents a single scFv/folate conjugate prepared using an EDC/folate ratio of 5:1 (EDC 33–65 mM) and tested in cytotoxicity assays using F2-MTX^{r}A and CTL 2C.

of amino acid residues important in TCR binding by the scFv or in scFv stability.

Comparison of Intact Antibody and scFv. To determine the relative effectiveness of intact KJ16 IgG versus scFv-KJ16, both forms were labeled with folate at a 100:1 molar ratio of activated folate to antibody and under identical EDC reaction conditions (33 mM EDC, 6.7 mM folate). These conditions yielded folate densities of 7 and 5 for the scFv and intact antibody, respectively. Cytotoxicity assays with these preparations showed nearly identical targeting efficiencies for the intact and scFv forms of KJ16 (Figure 6). The concentration required to obtain 50% of the maximal specific release (EC_{50}) was approximately 40 pM (1.2 ng/mL for scFv). Comparison of intact and scFv conjugates in an ^{125}I-labeled folate binding assay indicated no significant difference in their ability to bind FR (data not shown).

Intact KJ16 antibody was also labeled at various folate densities to determine if targeting efficiency could be optimized further. Conjugates were again evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency (Fig-

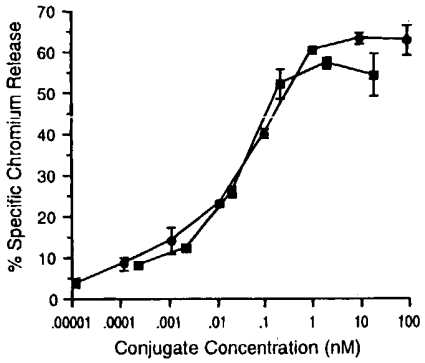

Figure 6. Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells. Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 mM EDC, 100:1 molar ratio of folate/antibody), yielding conjugates with 7 fol/scFv (●) and 5 fol/IgG (■). Cytotoxicity assays were performed with these conjugates using ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C.

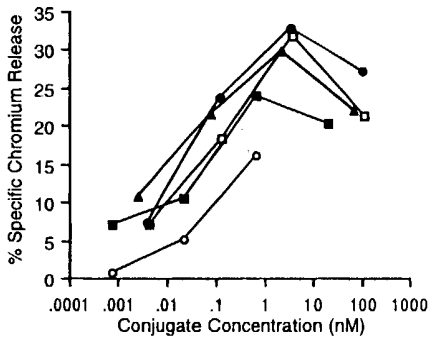

Figure 7. Effect of folate density on targeting with IgG/folate conjugates. Intact antibody was labeled at several folate concentrations to determine if, like scFv, a specific range of folate density would yield the optimal targeting effectiveness. Conjugates with the following densities were assayed with ^{51}Cr-labeled F2-MTX^{r}A cells and CTL clone 2C: 4.5 fol/IgG, □; 7.0 fol/IgG, ●; 13.6 fol/IgG, ▲; 56.7 fol/IgG, ■; and 126.2 fol/IgG, ○.

ure 7). Folate densities ranged from approximately 4−126 folates/antibody molecule. As shown with scFv preparations, high and low folate density resulted in decreased targeting effectiveness for the intact antibody. As with scFv preparations, the optimal densities appear to be in the range of 5−15 folates/antibody, as determined by spectrophotometry.

Inhibition of scFv/Folate Conjugate-Mediated Lysis by Free Folate. Normal serum folate may reduce the effectiveness of the scFv/folate conjugate by competing for the folate receptor *in vivo*. A ^{51}Cr release assay was used to evaluate the effectiveness of the scFv/folate conjugate-mediated lysis at biologically relevant concentrations of free folate (Figure 8). Free folate was diluted in folate-free media, and various concentrations were added to triplicate wells containing ^{51}Cr-labeled F2-MTX^{r}A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (3 nM) that would generate maximal specific release. The resulting titration curve demonstrated that at normal human serum folate levels [9−36 nM (*42*, *43*)] the scFv/folate conjugate retained most of its activity. For instance, at 20 nM folate, the scFv/folate conjugate exhibited over 80% of its CTL-mediated targeting potential. Although murine serum folate levels are significantly higher than normal human serum values (124−700 nM) due to folate-rich chow (*44*, *45*), even at these elevated folate concentrations, the scFv/folate conjugate exhibited 30−60% of its targeting potential.

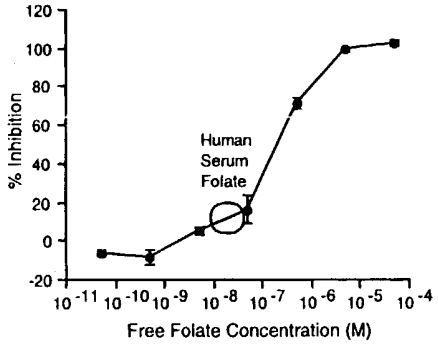

Figure 8. Inhibition of scFv/folate-mediated lysis by free folate. Various concentrations of free folate were added to triplicate wells containing ^{51}Cr-labeled F2-MTX^{r}A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (~8 fol/scFv) that would yield maximal specific release (3 nM). Inhibition was calculated as a function of the specific release in the absence of folate competitor. Normal human serum folate concentrations range from 9 to 36 nM (*42*, *43*).

DISCUSSION

The most effective agents for targeting solid tumors will likely have reduced sizes that allow greater tumor penetration. This paper characterizes the smallest bispecific agent yet described for redirecting the activity of immune effector cells against tumors. Initial bispecific antibody studies to target ovarian tumors that express high-affinity FR have used intact heterobifunctional antibodies (~150 kDa) that bind to CD3 and the FR. These agents showed efficacy in animal models, and they have recently entered testing in clinical trials (*9*, *10*, *12*). Although not yet reported for anti-FR antibodies, several laboratories have shown that it is possible to engineer smaller bispecific antibodies of ~60 kDa by linking two scFv regions (*41*, *46*, *47*). Here we show that the size of a bispecific targeting agent can be reduced even further to ~30 kDa for the scFv/folate conjugates. Furthermore, the targeting efficiency of the engineered scFv/folate conjugate is comparable to that of the native intact antibody/folate conjugates.

For coupling of folate to the anti-Vβ8 scFv KJ16, a carbodiimide reaction that links the carboxyl groups of folate to the amino groups of the antibody was used. KJ16 scFv binds to the cell surface TCR with an affinity that is similar to that of the intact KJ16 antibody (*29*). The V_L and V_H regions of the scFv are linked by a 26 amino acid region that contains 8 lysine residues. We reasoned that this charged linker would be accessible to folate, would be located distal to the binding site, and would contain multiple attachment sites for folate through the EDC reaction. Under coupling conditions where folate concentrations are nonsaturating, the scFv and intact antibody have comparable densities of folate attached per molecule (~5−15 folates/molecule), despite the 5-fold greater size of the intact antibody. Cytolytic assays demonstrated that these conjugates have very similar targeting efficiencies (Figure 6).

Although folate is probably attached to lysines present in the linker, the scFv/folate conjugates are actually heterogeneous populations as evidenced by mass spectra (Figure 1B). Within a scFv/folate preparation there are most likely conjugates with enhanced targeting properties and conjugates with diminished targeting properties. The former could include not only those with folate at an accessible location but perhaps those with multiple

folates that allow for multivalent interactions with FRs on tumor cells. Conjugates which have diminished bispecific properties would include those that have folate attached through amine groups in the scFv active site or in regions which destabalize the V_L–V_H interaction. These are analogous to those preparations derived with very high folate densities, where the targeting efficiency is significantly reduced (Figures 5 and 7).

Several considerations suggest that further optimization of the scFv/folate conjugates could yield even more potent agents. The anti-TCR antibody KJ16 has a relatively modest K_D of ~100 nM (*29*). Our laboratory is currently engineering higher affinity anti-TCR antibodies by antibody display methods; antibodies with $K_D \leq 1$ nM have been routinely generated using similar approaches (*48*). In addition, the affinity of the scFv/folate conjugate for the FR^+ tumor cells was up to 30-fold less than the affinity of free folate for the FR (Figure 3). Coupling of folate through the γ carboxyl, plus homogeneous linkage perhaps through cysteine or multiple cysteines, should improve the affinity for the FR. Alternatively, other folate analogs with higher affinity than folate could be employed (*49*). The use of these strategies should allow the development of scFv conjugates that have EC_{50} values considerably less than those described in this paper.

It is of significant note that the scFv agent described here not only is the smallest agent but is at least as potent *in vitro* as bispecific antibodies described in the literature. For example, the EC_{50} of other bispecific agents range from 1 to 100 ng/mL (*21, 50–52*). Various other antibody or folate-based targeting agents have EC_{50} values that range from 0.1 to 200 ng/mL (e.g. refs 37, 40, 53–56). *In vitro* assays for these other agents typically involved 24 h incubation periods, while the cytotoxicity assays for CTL-mediated lysis, described here and elsewhere, are 4 h incubations. Thus, there is reason to believe that the scFv/folate conjugate has considerable promise *in vivo*. It has an EC_{50} of approximately 1 ng/mL, is smaller than the other agents, and remains effective at folate levels found in normal human serum. The scFv/folate targeting effect was inhibitable by free folate, but only at concentrations that were considerably higher (>1000-fold) than the folate conjugate concentration (Figure 8). Schodin *et al.* (*57*) have previously shown that <1% of the total number of TCRs per CTL need to be triggered for CTL-mediated cytolysis to occur. Thus, it is possible that >90% of the folate/antibody conjugates were inhibited from binding the target cell but sufficient conjugate remained bound to trigger maximal lysis.

The fact that the scFv/folate conjugate is in direct competition with serum folate also brings about the intriguing possibility of modulating the effectiveness of scFv/folate conjugate treatment by altering the levels of serum folate. For instance, recent studies with mice have shown that serum folate can be intentionally decreased up to 100-fold with special low-folate diets (*44, 45*). The decreased folate concentration greatly enhanced the ability of a ^{67}Ga-labeled deferoxamine/folate conjugate to image FR^+ tumors *in vivo* (*44*). We envision that similar low-folate diets will likewise enhance the therapeutic effectiveness of the scFv/folate conjugate. Conversely, serum folate could be increased in situations where nonspecific T cell interactions lead to adverse side effects. Thus, the use of folate as the small molecule ligand specific for a tumor antigen may allow for additional levels of regulation normally not available with other immunotargeting agents.

In vivo tests to compare scFv and intact folate conjugates are currently underway in SV40 transgenic mice that develop choroid plexus tumors exhibiting elevated levels of the high affinity folate receptor (*58*). Preliminary results indicate that T cells specifically infiltrate the tumor after treatment with the scFv/folate conjugate (B.K.C., T.A.P., D.M.K., and E.J.R., unpublished data). At this time, it is unclear whether conjugate-bound T cells extravasate into the tumor or if T cells first extravasate into the tumor and subsequently recognize the conjugate bound to cell surface FR. The latter mechanism would favor the enhanced tumor penetration characteristics of the smaller scFv molecule and would likely lead to increased therapeutic effectiveness.

ACKNOWLEDGMENT

We thank Drs. John Kappler and Philippa Marrack for providing the KJ16 hybridoma line, Carol Schlueter for providing the anti-L^d ascites fluid, and Drs. Brigle, Spinella, and Goldman for the DBA/2 tumor cell lines. This work was supported by grants from the University of Illinois Research Board (E.J.R.), the National Institutes of Health (AI35990, E.J.R. and D.M.K.), the Department of the Army (DAMD17-94-J-4347, D.M.K.), and the National Institutes of Mental Health (MH11189-01, T.A.P.). The TofSpec mass spectrometer was purchased in part with Grant RR07141 from the Division of Research Resources, National Institutes of Health. Mass spectrometry was performed by Nelson Huang.

LITERATURE CITED

(1) Gorer, P. A. (1937) The genetic and antigenic basis of tumor transplantation. *J. Pathol. Bacteriol. 44*, 691–697.
(2) Boon, T. (1993) Teaching the immune system to fight cancer. *Sci. Am. 268*, 82–89.
(3) Pardoll, D. M. (1994) A new look for the 1990s. *Nature 369*, 357–358.
(4) Klein, G., and Boon, T. (1993) Tumor immunology: present perspectives. *Curr. Opin. Immunol. 5*, 687–692.
(5) Fanger, M. W., Morganelli, P. M., and Guyre, P. M. (1992) Bispecific antibodies. *Crit. Rev. Immunol. 12*, 101–124.
(6) Liu, M. A., Nussbaum, S. R., and Eisen, H. N. (1988) Hormone conjugated with antibody to CD3 mediates cytotoxic T cell lysis of human melanoma cells. *Science 239*, 395–398.
(7) Nitta, T., Yagita, H., Sato, K., Okumura, K., and Ishii, S. (1990) Preliminary trials of specific targeting therapy against malignant glioma. *Lancet 335*, 368–371.
(8) Chen, J., Zhou, J. H., Mokotoff, M., Fanger, M. W., and Ball, E. D. (1995) Lysis of small cell carcinoma of the lung (SCCL) cells by cytokine-activated monocytes and natural killer cells in the presence of bispecific immunoconjugates containin a gastrin-releasing peptide (GRP) analog or a GRP antagonist. *J. Hematother. 4*, 369–376.
(9) Canevari, S., Miotti, S., Bottero, F., Valota, O., and Colnaghi, M. I. (1993) Ovarian carcinoma therapy with monoclonal antibodies. *Hybridoma 12*, 501–507.
(10) Canevari, S., Stoter, G., Arienti, F., Bolis, G., Colnaghi, M. I., Di Re, E. M., Eggermont, A. M. M., Goey, S. H., Gratama, J. W., Lamers, C. H. J., Nooy, M. A., Parmiani, G., Raspagliesi, F., Ravagnani, F., Scarfone, G., Trimbos, J. B., Warnaar, S. O., and Bolhuis, R. L. H. (1995) Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J. Natl. Cancer Inst. 87*, 1463–1469.
(11) Weiner, L. M., Clark, J. I., Davey, M., Li, W. S., Garcia de Palazzo, I., Ring, D. B., and Alpaugh, R. K. (1995) Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. *Cancer Res. 55*, 4586–4593.
(12) Lamers, C. H., Gratama, J. W., Warnaar, S. O., Stoter, G., and Bolhuis, R. L. (1995) Inhibition of bispecific monoclonal antibody (bsAb)-targeted cytolysis by human anti-mouse antibodies in ovarian carcinoma patients treated with bsAb-targeted activated T-lymphocytes. *Int. J. Cancer. 60*, 450–457.

(13) Buist, M. R., Molthoff, C. F. M., Kenemans, P., and Meijer, C. J. L. M. (1995) Distribution of OV-TL 3 and MOv18 in normal and malignant ovarian tissue. *J. Clin. Pathol. 48*, 631–636.
(14) Ross, J. F., Chaudhuri, P. K., and Ratnam, M. (1994) Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer 73*, 2432–2443.
(15) Weitman, S. D., Frazier, K. M., and Kamen, B. A. (1994) The folate receptor in central nervous system malignancies of childhood. *J. Neurooncol. 21*, 107–112.
(16) Knapp, R. C., and Berkowitz, R. S. (1993) *Gynecologic Oncology*, 250 pp, McGraw-Hill, New York.
(17) Allen, J., Wisoff, J., Helson, L., Pearce, J., and Arenson, E. (1992) Choroid plexus carcinoma—responses to chemotherapy alone in newly diagnosed young children. *J. Neuro-Oncol. 12*, 69–74.
(18) Dohrmann, G. J., Farwell, J. R., and Flannery, J. T. (1976) Ependymomas and ependymoblastomas in children. *J. Neurosurg. 45*, 273–283.
(19) Coney, L. R., Tomassetti, A., Carayannopoulos, L., Frasca, V., Kamen, B. A., Colnaghi, M. I., and Zurawski, V. R., Jr. (1991) Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. *Cancer Res. 51*, 6125–6132.
(20) van Ravenswaay Claasen, H. H., van de Griend, R. J., Mezzanzanica, D., Bolhuis, R. L., Warnaar, S. O., and Fleuren, G. J. (1993) Analysis of production, purification, and cytolytic potential of bi-specific antibodies reactive with ovarian-carcinoma-associated antigens and the T-cell antigen CD3. *Int. J. Cancer 55*, 128–136.
(21) Coney, L. R., Mezzanzanica, D., Sanborn, D., Casalini, P., Colnaghi, M. I., and Zurawski, V. R. (1994) Chimeric murine human antibodies directed against folate binding receptor are efficient mediators of ovarian carcinoma cell killing. *Cancer Res. 54*, 2448–2455.
(22) Leamon, C. P., and Low, P. S. (1994) Selective targeting of malignant cells with cytotoxin-folate conjugates. *J. Drug Target. 2*, 101–112.
(23) Wang, S., Lee, R. J., Cauchon, G., Gorenstein, D. G., and Low, P. S. (1995) Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. *Proc. Natl. Acad. Asci. U.S.A. 92*, 3318–3312.
(24) Weitman, S. D., Lark, R. H., Coney, L. R., Fort, D. W., Frasca, V., Zurawski, V. R., Jr., and Kamen, B. A. (1992) Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res. 52*, 3396–3401.
(25) Weitman, S. D., Weinberg, A. G., Coney, L. R., Zurawski, V. R., Jennings, D. S., and Kamen, B. A. (1992) Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis. *Cancer Res. 52*, 6708–6711.
(26) Antony, A. C. (1996) Folate receptors. *Annu. Rev. Nutr. 16*, 501–521.
(27) Kranz, D. M., Patrick, T. A., Brigle, K. E., Spinella, M. J., and Roy, E. J. (1995) Conjugates of folate and anti-T cell receptor antibodies specifically target folate-receptor-positive tumor cells for lysis. *Proc. Natl. Acad. Sci. U.S.A. 92*, 9057–9061.
(28) Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res. 52*, 3402–3408.
(29) Cho, B. K., Schodin, B. A., and Kranz, D. M. (1995) Characterization of a single-chain antibody to the β-chain of the T cell receptor. *J. Biol. Chem. 270*, 25819–25826.
(30) Friend, C., Patuleia, M. C., and De Harven, E. (1966) Erythrocytic maturation *in vitro* of murine (Friend) virus-induced leukemic cells. *NCI Monographs No. 22*, 505–522.
(31) Brigle, K. E., Spinella, M. J., Westin, E. H., and Goldman, I. D. (1994) Increased expression and characterization of two distinct folate binding proteins in murine erythroleukemia cells. *Biochem. Pharmacol. 47*, 337–345.
(32) Law, L. W., B, D. T., Boyle, P. J., and Miller, J. H. (1949) Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. *J. Natl. Cancer Inst. 10*, 179–192.
(33) Brigle, K. E., Seither, R. L., Westin, E. H., and Goldman, I. D. (1994) Increased expression and genomic organization of a folate-binding protein homologous to the human placental isoform in L1210 murine leukemia cell lines with a defective reduced folate carrier. *J. Biol. Chem. 269*, 4267–4272.
(34) Kranz, D. M., Sherman, D. H., Sitkovsky, M. V., Pasternack, M. S., and Eisen, H. N. (1984) Immunoprecipitation of cell surface structure of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc. Natl. Acad. Sci. U.S.A. 81*, 573–577.
(35) Haskins, K., Hannum, C., White, J., Rhoem, N., Kubo, R., Kappler, J., and Marrack, K. (1984) The antigen-specific major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. *J. Exp. Med. 160*, 452–471.
(36) Ozato, K., Hansen, T. H., and Sachs, D. H. (1980) Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2L^d antigen, the product of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol. 125*, 2473–2477.
(37) Leamon, C. P., and Low, P. S. (1992) Cytotoxicity of momordin-folate conjugates in cultured human cells. *J. Biol. Chem. 267*, 24966–24971.
(38) Whitlow, M., and Filpula, D. (1991) Single-chain Fv proteins and their fusion proteins. *Methods: A Companion to Methods in Enzymology*, Vol. 2, pp 97–105, Academic, San Diego.
(39) Rojo, J. M., and Janeway, C. A., Jr. (1988) The biologic activity of anti-T cell receptor V region monoclonal antibodies is determined by the epitope recognized. *J. Immunol. 140*, 1081–1088.
(40) Leamon, C. P., Pastan, I., and Low, P. S. (1993) Cytotoxicity of folate-*Pseudomonas* exotoxin conjugates toward tumor cells. Contribution of translocation domain. *J. Biol. Chem. 268*, 24847–24854.
(41) Gruber, M., Schodin, B., Wilson, E., and Kranz, D. M. (1994) Efficient tumor cell lysis mediated by a bispecific single-chain antibody expressed in *E. coli*. *J. Immunol. 152*, 5368–5374.
(42) Kutsky, R. J. (1981) *Handbook of Vitamins, Minerals, and Hormones*, 2nd ed., 492 pp, Van Nostrand Reinhold, New York.
(43) Lentner, C. (1984) *Geigy Scientific Tables, 8th rev., Physical chemistry, composition of blood, hematology, somatometric data*, Vol. 3, 131 pp, Ciba-Geigy Limited, Basle, Switzerland.
(44) Mathias, C. J., Wang, S., Lee, R. J., Waters, D. J., Low, P. S., and Green, M. A. (1996) Tumor-selective radiopharmaceutical targeting via receptor-mediate endocytosis of gallium-67-deferoxamine-folate. *J. Nucl. Med. 37*, 1003–1008.
(45) Gospe Jr., S. M., Gietzen, D. W., Summers, P. J., Lunetta, J. M., Miller, J. W., Selhub, J., Ellis, W. G., and Clifford, A. J. (1995) Behavioral and neurochemical changes in folate-deficient mice. *Physiol. Behav. 58*, 935–941.
(46) Hollinger, P., Prospero, T., and Winter, G. (1993) "Diabodies": Small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. U.S.A. 90*, 6444–6448.
(47) Kurucz, I., Titus, J. A., Jost, C. R., Jacobus, C. M., and Segal, D. M. (1995) Retargeting of CTL by an efficiently refolded bispecific single-chain Fv dimer produced in bacteria. *J. Immunol. 154*, 4576–4582.
(48) Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) Making antibodies by phage display technology. *Annu. Rev. Immunol. 12*, 433–455.
(49) Elwood, P. C., Kane, M. A., Portillo, R. M., and Kolhouse, J. F. (1986) The isolation, characterization, and comparison of the membrane-associated and soluble folate-binding proteins from human KB cells. *J. Biol. Chem. 261*, 15416–15423.
(50) Mack, M., Riethmuller, G., and Kufer, P. (1995) A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc. Natl. Acad. Sci. U.S.A. 92*, 7021–7025.
(51) Zhu, Z., Zapata, G., Shalaby, R., Snedecor, B., Chen, H., and Carter, P. (1996) High level secretion of a humanized bispecific diabody from *Escherichia coli*. *Biotechnology 14*, 192–196.

(52) Sugiyama, Y., Aihara, M., Shibamori, M., Deguchi, K., Imagawa, K., Kikuchi, M., Momata, H., Azuma, T., Okada, H., Alper, Ö., Hitomi, J., and Yamaguchi, K. (1992) *In vitro* anti-tumor activity of anti-c-*erb*B-2 x anti-CD3ϵ bifunctional monoclonal antibody. *Jpn. J. Cancer Res. 83*, 563−267.

(53) Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., and Pastan, I. (1989) A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature 339*, 394−397.

(54) Chaudhary, V. K., Gallo, M. G., FitzGerald, D. J., and Pastan, I. (1990) A recombinant single-chain immunotoxin composed of anti-Tac variable regions and a truncated diphtheria toxin. *Proc. Natl. Acad. Sci. U.S.A. 87*, 9491−9494.

(55) Burrows, F. J., and Thorpe, P. E. (1993) Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature. *Proc. Natl. Acad. Sci. U.S.A. 90*, 8996−9000.

(56) Wels, W., Harwerth, I. M., Mueller, M., Groner, B., and Hynes, N. E. (1992) Selective inhibition of tumor cell growth by a recombinant single-chain antibody-toxin specific for the erbB-2 receptor. *Cancer Res. 52*, 6310−6317.

(57) Schodin, B. A., Tsomides, T. J., and Kranz, D. M. (1996) Correlation between the number of T cell receptors required for T cell activation and TCR-ligand affinity. *Immunity 5*, 137−146.

(58) Patrick, T. A., Kranz, D. M., Van Dyke, T. A., and Roy, E. J. (1997) Folate receptors as potential therapeutic targets in choroid plexus tumors of SV40 transgenic mice. *J. Neuro-Oncol.* (in press).

BC9700244